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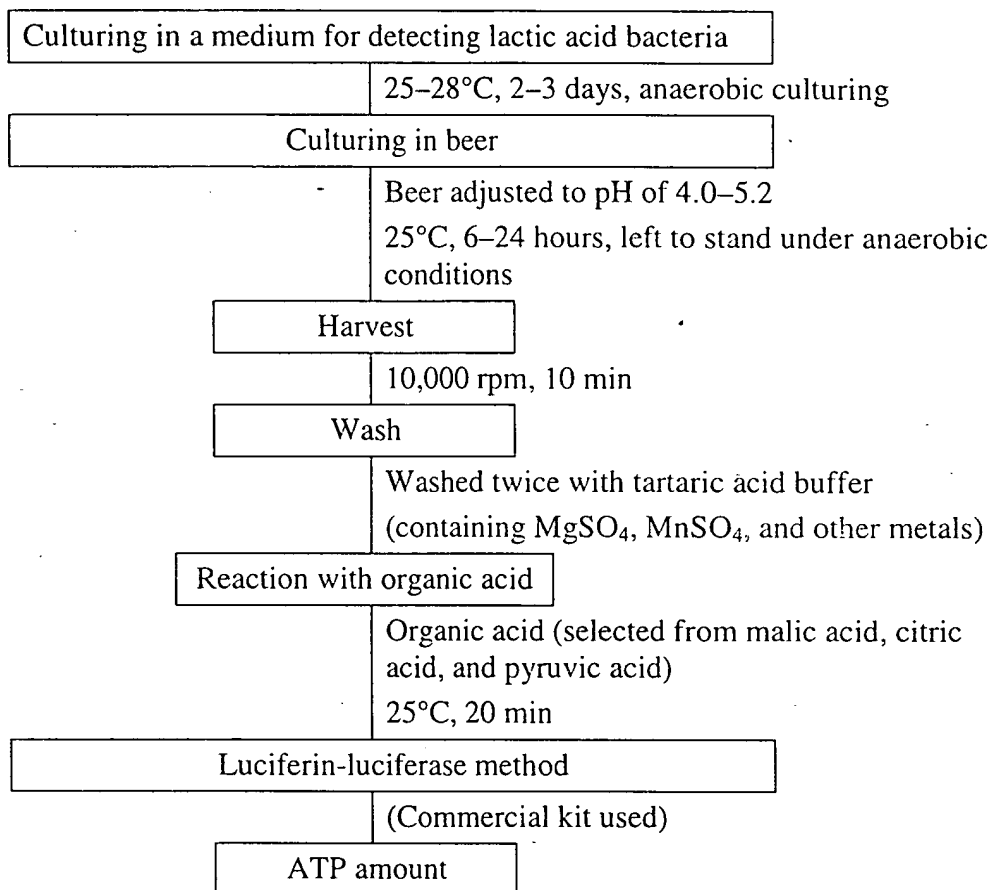
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(54) [Title of the Invention] Method for the Early Determination of Lactic Acid  
Bacteria That Grow in Beer, and a Differentiation Kit

(57) [Summary]

[Object] To provide a method that allows for the determination at an early stage of whether lactic acid bacteria that are detected grow in beer, and to provide a differentiation kit for the same.

**[Means of Achievement]** A method for the early determination of lactic acid bacteria that grow in beer, characterized in that the lactic acid bacteria to be identified are reacted with an organic acid, and the amount of ATP in the bacterial cells is then measured by the luciferin-luciferase method.



**[Claims]**

**[Claim 1]** A method for the early determination of lactic acid bacteria that grow in beer, characterized in that the lactic acid bacteria to be identified are reacted with an organic acid, and the amount of ATP in the bacterial cells is then measured by means of the luciferin-luciferase method.

**[Claim 2]** The determination method according to claim 1, wherein the organic acid comprises one or more types selected from pyruvic acid, malic acid, and citric acid.

**[Claim 3]** A method for the early determination of lactic acid bacteria that grow in beer, characterized in that the lactic acid bacteria to be identified are added to and cultured in a medium for detecting lactic acid bacteria, one or more organic acids selected from pyruvic acid, malic acid, and citric acid are added, a reaction is carried out, and the amount of ATP in the bacterial cells is measured by means of the luciferin-luciferase method.

**[Claim 4]** The determination method according to claim 3, wherein beer or hop components are added to the medium for detecting lactic acid bacteria.

**[Claim 5]** A kit for detecting lactic acid bacteria that grow in beer, comprising a luciferin-luciferase luminescent solution, an ATP extract, an organic acid reaction solution, a beer solution for culturing, and a culture medium for detecting lactic acid bacteria.

#### **[Detailed Description of the Invention]**

**[0001]**

**[Technological Field of the Invention]** This invention relates to a method for the early determination of lactic acid bacteria that grow in beer, and a differentiation kit for the same.

**[0002]**

**[Prior Art]** When beverages and foodstuffs are contaminated with harmful microorganisms, the product quality is markedly affected by turbidity, acidification, and the like. In the case of beer, the gram-positive *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and other types of lactic acid bacteria are the typical bacteria detected as beer contaminants. Not all these bacterial strains necessarily grow in beer and compromise beer quality, however. There are bacteria that belong to the same genus and that can grow [in beer as well], bacteria that can grow only after becoming acclimated to beer, and bacteria completely incapable of growing in beer. Therefore, if bacterial strains belonging to these genera are detected in beer products and beer manufacturing processes, evaluating the possibility of their growth in beer is extremely important.

**[0003]** Lactic acid bacteria detected in manufacturing processes, beer products, or the like are currently reseeded in beer from a detecting agar medium, and their turbidity and bacterial count are measured to determine growth. This approach is disadvantageous in that not all the lactic acid bacteria will necessarily grow and that considerable time (about 1-2 months) elapses until

such growth is determined. It is also known that ATP or the luciferin-luciferase method is used to identify beer-spoiling lactic acid bacteria. Main examples of references in which such use is described include "Lactic acid bacteria assay method and kit" (JP (Kokai) 54-123049), "Method for measuring *Bacillus saprogenes*" (JP (Kokai) 07-195), "Measuring bacterial count of latent beer-spoiling bacteria by ATP bioluminescence" (*J. Am. Soc. Brew.*, Vol. 52, No. 1, 19-23 (1994)), and "Rapid method for detecting yeast and lactic acid bacteria by ATP bioluminescence" (*J. Inst. Brew.*, Vol. 95, No. 1, 317-319 (1989)). In these methods, the ATP in the harmful lactic acid bacterial cells is made to emit light by the luciferin-luciferase method, and the presence of the corresponding bacteria is determined based on the intensity of such luminescence, and the bacterial count thereof is measured if such a presence is confirmed.

[0004]

**[Problems Which the Invention Is Intended to Solve]** The conventionally used techniques of measuring the ATP in lactic acid bacterial cells by the luciferin-luciferase method primarily only entail determining the presence of lactic acid bacteria and measuring the bacterial count of the lactic acid bacteria by extracting the ATP present in the lactic acid bacterial cells and measuring the ATP thereof, but these techniques are incapable of determining whether the lactic acid bacteria thus detected can grow in beer.

[0005] Accordingly, this invention provides a method and kit for the early determination of whether detected lactic acid bacteria can grow in beer.

[0006]

**[Means for Solving the Problem]** Directed at accomplishing the intended object, this invention provides a determination method and kit in which the lactic acid bacteria to be identified are cultured, one or more organic acids selected from pyruvic acid, malic acid, and citric acid are added, a reaction is carried out for a predetermined time; and the amount of ATP in the bacterial cells is then measured by means of the luciferin-luciferase method.

[0007] Lactic acid bacteria that grow in beer consume the pyruvic acid, malic acid, or citric acid in the beer, and start to generate lactic acid, acetic acid, or carbon dioxide gas. In the case of lactic acid bacteria that do not grow in beer, the pH of the bacterial cells is reduced by the ionophoric action of the isohumulone, and the bacteria are inhibited in their growth and are

destroyed. Focusing on this aspect, ATP production was measured based on the decarboxylation of organic acids such as pyruvic acid, malic acid, and citric acid by employing lactic acid bacteria that grow in beer and lactic acid bacteria that do not grow there, whereupon it was confirmed that the amount of the ATP generated in the bacterial cells that do grow in beer was significantly higher compared with the amount found in the non-growth bacteria.

[0008] In view of the above, this phenomenon is used in the present invention in such a manner that malic acid or another organic acid is reacted with the lactic acid bacteria of a sample, the amount of ATP in the lactic acid bacterial cells being identified is varied, the varying amount of ATP is measured by means of the luciferin-luciferase method, which is a known technique, and the ability of the lactic acid bacteria to grow in beer is determined. In this process, the amount of ATP in the bacterial cells increases in the case of lactic acid bacteria that grow in beer, whereas the amount of ATP in the bacterial cells does not increase in the case of non-growth lactic acid bacteria.

[0009] With this method, it is possible to predict at an early stage (6–24 hours) whether the bacteria detected in beer products or beer-making processes can grow in beer.

[0010]

**[Embodiments of the Invention]** This invention can be carried out as follows. The lactic acid bacteria to be identified are first cultured in a medium for detecting lactic acid bacteria. The culturing is carried out anaerobically for 2–3 days under anaerobic conditions at a pH of 4.5–6.0 and a temperature of 25–28°C. No restrictions are imposed on the culture medium for detecting lactic acid bacteria as long as it provides conditions conducive to moderate growth of lactic acid bacteria in beer, although 10–50% of beer may be added to the culture medium to accelerate the growth. It is also possible to add a hop component (2–10 ppm, isohumulone), organic acids (pyruvic acid, malic acid, citric acid), and metals (0.5–1.5 mM MgSO<sub>4</sub>, MnSO<sub>4</sub>, and the like).

[0011] The lactic acid bacteria thus cultured are subsequently harvested and washed with a buffer. For improved detection efficiency, a step in which culturing is performed for 6–24 hours with beer at a pH of 4.0–5.2 may be added following harvesting. The washed lactic acid bacteria are suspended in an organic acid solution and reacted for 2–30 minutes. The optimal reaction

duration may be set depending on the temperature conditions of the actual operation. The organic acid may be malic acid, citric acid, pyruvic acid, or the like. The organic acid should preferably be used at a concentration of 1–20 mM. The amount in which lactic acid bacteria are added to the organic acid solution should fall within a range of  $10^3$ – $10^{10}$  cells/mL. The reacted lactic acid bacteria are measured by means of the known luciferin-luciferase method, and the amount of ATP in the bacterial cells is determined. The luciferin-luciferase method is easy to use by employing a conventional measurement kit or the like.

[0012] A system devoid of an added organic acid is used as the control, and the amount of ATP in the bacterial cells is similarly determined. The ATP amount thus obtained is substituted into the following equation.

$$\frac{\text{Amount of ATP in bacterial cells with added organic acid}}{\text{Amount of ATP in bacterial cells without added organic acid (value of control test)}}$$

If the resulting value is 2 or greater, it is concluded that growth in beer is possible. The flow chart of the method of this invention is shown in Fig. 1.

[0013] Based on the determination method described above, it is also possible to produce a kit for detecting lactic acid bacteria that grow in beer. Its composition is shown in Table 1.



[0014]

[Table 1]

1. Medium for detecting lactic acid bacteria	
① Composition of medium for growing lactic acid bacteria commonly used to detect lactic acid bacteria or the like	
(Example) Culture components of MRS broth, manufactured by Merck	
peptone	10 (g/L)
meat extract	8
yeast extract	4
glucose	20
potassium hydrogen phosphate	2
ammonium hydrogen citrate	2
sodium acetate	5
magnesium sulfate	0.2
manganese sulfate	0.04
② malate	1.5–3.5 (g/L)
③ isohumurone	2–10 ppm
Items ①–③ are mixed, and the pH is adjusted to 4.5–5.5	
2. Luciferin-luciferase luminescent solution	
Solution A: 1–0.10 ng/ $\mu$ L	luciferase (8 mM MgCl)
Solution B: 2–5 mg/mL	luciferin
Solutions A and B are dissolved in HEPES buffer at a pH of 7.7	
3. Incubation solution	
Beer adjusted to pH of 4.0–5.0	
4. Organic acid reaction solution	
1–20 mM of organic acid (selected from malic acid, citric acid, and pyruvic acid) is added to and dissolved in a tartaric acid buffer containing 0.5–1.5 mM of metal (MgSO <sub>4</sub> , 0.5 mM MnSO <sub>4</sub> , etc.) and having an adjusted pH of 4.0–5.0	
5. ATP extract (liquid components commonly employed as ATP extract may be used)	
pH 7.8, 25 mM Tris phosphate buffer	
1 mM dithiothreitol	
1.6 mM ethylenediaminetetraacetic acid	
1.6% Triton X-100	
1% bovine serum albumin	
15% glycerol	

[0015]

[Merits of the Invention] According to this invention, lactic acid bacteria that grow in beer can be identified at an early stage by a simple method.

[0016]

[Practical examples] This invention is described below with the help of practical examples, but is not limited thereby.

### Practical Example 1

Seven types of bacterial strains that grow in beer (*Lactobacillus brevis* Asahi Beer Bacteria Collection (referred to hereinbelow as "ABBC") 45, *Lactobacillus brevis* ABBC46, *Lactobacillus brevis* ABBC65, *Lactobacillus brevis* ABBC69, *Lactobacillus sp.* ABBC70, *Lactobacillus brevis* ABBC99, and *Lactobacillus brevis* ABBC104) and seven types of bacterial strains that do not grow in beer (*Lactobacillus brevis* ABBC216, *Lactobacillus buchneri* ABBC219, *Lactobacillus buchneri* ABBC220, *Lactobacillus buchneri* ABBC221, *Lactobacillus paracasei* ABBC223, *Lactobacillus sp.* ABBC229, and *Lactobacillus sp.* ABBC232) were suspended in 10 mL each of a medium for detecting lactic acid bacteria (trade name: MRS broth; manufactured by Merck ) and in a beer-containing MRS medium that was obtained by adding 25% of beer to the MRS medium for detecting lactic acid bacteria (referred to hereinbelow as "BMRS medium"). This was followed by anaerobically culturing for 3 days at 25°C. A 0.1% solution of the cultured broth was added to 10 mL of beer that had been adjusted to a pH of 5.0, and the product was cultured for 24 hours at 25°C. Each bacterial suspension was centrifuged for 10 minutes at 10,000 rpm, and the product was harvested and washed with a tartaric acid buffer at a pH of 4.2. The product was then centrifuged, the supernatant was removed, the biomass was again suspended in the tartaric acid buffer at the pH of 4.2, 20 mM of malic acid and 1 mM of citric acid were added as substrates to the suspension, and a reaction was run for 20 minutes at 25°C to generate ATP in the bacterial cells.

[0017] The ATP in the bacterial cells was measured by the luciferin-luciferase method using the Kikkoman kit (trade name: Luciferase LU plus). As a control, a product devoid of any added organic acid was reacted in an identical system, and the growth ability was determined using the equation shown below. Growth was deemed possible if the following condition was met.

$$\frac{\text{Amount of ATP in bacterial cells with added organic acid}}{\text{Amount of ATP in bacterial cells without added organic acid}} > 2$$

[0018] When the amount of ATP in bacterial cells was determined, it was possible to clearly distinguished between bacteria that grow in beer and bacteria that do not grow in beer within a short time. The results are presented in Table 2.

[0019]

[Table 2]

	ATP production (relative value)		
	Without organic acid added	With organic acid added	
		MRS medium	Beer-added MRS medium
Bacteria that grow in beer			
<i>Lactobacillus brevis</i> ABBC45	1.00	1	9
<i>Lactobacillus brevis</i> ABBC46	1.00	2	7
<i>Lactobacillus brevis</i> ABBC65	1.00	1	5
<i>Lactobacillus brevis</i> ABBC69	1.00	2	14
<i>Lactobacillus</i> sp. ABBC70	1.00	2	5
<i>Lactobacillus brevis</i> ABBC99	1.00	1	8
<i>Lactobacillus brevis</i> ABBC104	1.00	2	8
Bacteria that do not grow in beer			
<i>Lactobacillus brevis</i> ABBC216	1.00	1	1
<i>Lactobacillus buchneri</i> ABBC219	1.00	1	1
<i>Lactobacillus buchneri</i> ABBC220	1.00	1	1
<i>Lactobacillus buchneri</i> ABBC221	1.00	1	1
<i>Lactobacillus paracasei</i> ABBC223	1.00	1	1
<i>Lactobacillus</i> sp. ABBC229	1.00	1	1
<i>Lactobacillus</i> sp. ABBC232	1.00	1	1

[Brief Description of Drawings]

[Fig. 1] A flowchart of the early determination method for lactic acid bacteria that grow in beer.

[Fig. 1]

